Non-Radioisotopic Kit for Measuring PKR/EIF2AK2 Activity

CycLex PKR/EIF2AK2 Kinase Assay Kit

Cat# CY-1184

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Intended Use

The CycLex Research Product **CycLex PKR/EIF2AK2 Kinase Assay Kit** is primarily designed to measure the activities of purified RNA-dependent protein kinase (PKR) or recombinant PKR for the rapid and sensitive evaluation of inhibitors or activators. The phospho-serine specific monoclonal antibody used in this assay kit has been demonstrated to recognize the phosphorylated serine 51 in Eukaryotic Initiation Factor 2α (eIF2α), which is efficiently phosphorylated by PKR. Additionally, column fractions of cultured primary cells, cell lines, or tissues can be assayed for PKR activity with the CycLex Research Product **CycLex PKR/EIF2AK2 Kinase Assay Kit** if the appropriate dose of PKR specific inhibitor, e.g. C16, is used.

Applications of this kit include:
1) Screening inhibitors or activators of PKR/EIF2AK2.
2) Evaluating the effects of pharmacological agents on PKR/EIF2AK2 activity *in vitro*.
3) Monitoring the purification of the kinase activity of PKR/EIF2AK2.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Don't expose reagents to excessive light.

For Reference Purpose Only: Please refer to the user manual that came with your product.

Introduction

The double-stranded RNA-activated protein kinase (PKR), also known as eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2), is a ubiquitously expressed serine/threonine protein kinase that plays a key role in the innate immunity response to viral infection in higher eukaryotes and has also been implicated in several cellular signal transduction pathways (1-4).

The dsRNAs-mediated activation leads to autophosphorylation of PKR and allows the kinase to phosphorylate its natural substrate, the α-subunit of initiation factor eIF2, resulting in rapid inhibition of translation and suppression of virus spread (5, 6). PKR also has been implicated in regulating other cellular functions such as differentiation (7), transcription (8, 9), signal transduction (10), cell growth (11, 12) and apoptosis in the event of virus infection and other forms of cellular stress (13-15).

It was reported that the activation of PKR in adipose and liver tissue is caused by obesity (16). In the absence of PKR, metabolic deterioration due to excess energy or nutrition is alleviated. These findings demonstrate that PKR is an important component of inflammation complex that responds to nutrients and organelle dysfunction.

Principle of the Assay

The CycLex Research Product **CycLex PKR/EIF2AK2 Kinase Assay Kit** is a semi-quantitative immunoassay for the kinase activity of PKR. This product can be used to determine the presence of the kinase activity in purification column fractions or to follow the kinetics of a purified or partially purified PKR protein as well as screening PKR inhibitors or activators.

The protocol for the quantitative measurement of the kinase activity involves incubation of the PKR sample with its substrate, GST-eIF2α fusion protein, in the presence of $Mg^{2+}$and ATP, followed by transfer this kinase reaction mixture to the well of microtiter plate, which is pre-coated with a monoclonal antibody specific for phospho-eIF2α-S51 for trapping only phosphorylated substrate. The amount of phosphorylated substrate on the well is measured by a horseradish peroxidase coupled anti-GST antibody, which then catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent). The color is quantified by spectrophotometry and reflects the relative amount of the kinase activity in the sample.

For kinetic analysis, the sample containing PKR is added to the wells in a similar fashion and at varying times the reaction is stopped by the addition of a chelator, sodium ethylenediaminetetraacetate (EDTA) and the amount of phosphorylated substrate determined as mentioned before.

The CycLex Research Product CycLex PKR/EIF2AK2 Kinase Assay Kit is designed to accurately determine the presence and relative amount of PKR activity in purification column fractions and to determine non-isotopic kinetic analysis of the kinase activity of PKR. Careful attention to extraction methods and the assay protocol will provide the investigator with a reliable tool for the evaluation of the kinase activity.

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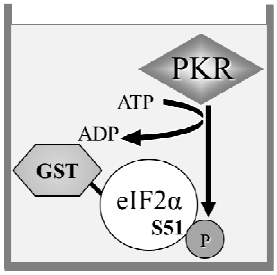
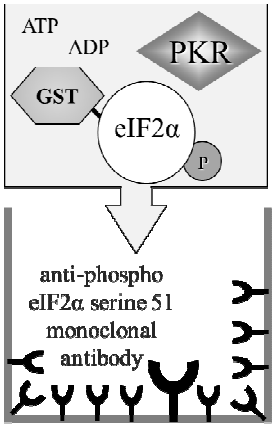
Summary of Procedure



Add 100 µL of reaction mixtures to the non-coated wells.

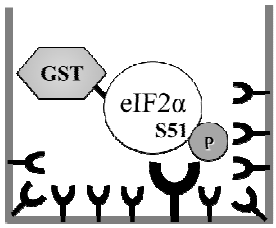
Incubate for 1 hour at 30°C.

Add of 10 µL of EDTA Solution to stop kinase reaction.



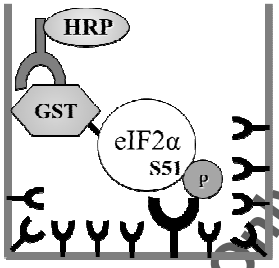
Transfer 100 µL of reaction mixtures to the antibody-coated wells.

Incubate for 1 hour at room temp.



Wash the wells.

Add 100 µL of HRP conjugated anti-GST antibody.



Incubate for 1 hour at room temp.

Wash the wells.

Add 100 µL of Substrate Reagent.

Incubate for 5 to 20 minutes at room temp.

Add 100 µL of Stop Solution.

Measure absorbance at 450 nm.

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Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate kit.

Non-coated Microplate for kinase reaction**:** One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a clear, zip-lock bag.

Antibody-coated Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with anti-phospho-eIF2α serine 51 monoclonal antibody as a capture antibody.

10X Wash Buffer: One bottle containing 100 mL of 10X buffer containing Tween®-20

Kinase Buffer: One bottle containing 20 mL of 1X buffer, used for Reaction Buffer and sample dilution.

20X ATP: One vial of lyophilized ATP Na_2 salt.

20X DTT: Four vials of lyophilized dithiothreitol.

10X GST-eIF2α Substrate: One vial containing 37.5 ng of lyophilized recombinant GST-eIF2α.

EDTA Solution: One vial containing 2 mL of 0.5 M EDTA, pH 8.0. Ready to use.

HRP conjugated Detection Antibody: One bottle containing 12 mL of HRP (horseradish peroxidase) conjugated anti-GST antibody. Ready to use.

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle containing 20 mL of 1 N H_2SO_4. Ready to use.

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Materials Required but not Provided

- **Orbital microplate shaker**
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1000 µL precision pipettors with disposable tips
- **Wash bottle or multichannel dispenser** for plate washing.
- **Microcentrifuge and tubes** for sample preparation
- **Vortex mixer**
- **Microplate washer:** optional (Manual washing is possible but not preferable)
- **Plate reader:** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**
- **PKR/EIF2 (Catalytic domain):** Available from CycLex Co., Ltd., PKR/EIF2AK2 (Human), Active, Cat#CY-SPP80
- **PKR/EIF2 (Full length):** Available from CycLex Co., Ltd., PKR/EIF2AK2 Positive Control (Full length), Cat#CY-E1184-1
- **(Optional) BSA-containing dilution buffer:** 20mM Hepes-KOH (pH 7.5), 1% BSA, 1mM EDTA, 2mM DTT, 50mM NaCl and 50% glycerol
- **(Optional) C16 (10 µM):** A specific inhibitor of PKR. Available from Sigma, Cat#I9785. Make 1 mM stock solution with DMSO and dilute 1:100 with Kinase Buffer.
- **(Optional) Poly I:C (Polyinosinic-polycytidylic acid sodium salt):** A synthetic analog of double-stranded RNA (dsRNA). Available from Sigma, Cat#P1530
- **(Optional) Phosphatase inhibitor cocktail:** Available from Cell Signaling Technology, Inc., Cat#5871
- **(Optional) Anti-PKR antibody:** Available from CycLex Co., Ltd., Cat#CY-P1043

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Precautions and Recommendations

- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents in this kit may contain preservatives or other chemicals. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of rat origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

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Detailed Protocol

The **CycLex PKR/EIF2AK2 Kinase Assay Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since conditions may vary, running an aliquot of the appropriate PKR Positive Control should be included in each assay. Disposable pipette tips and reagent troughs should be used for all transfers to avoid cross-contamination of reagents or samples.

1. Preparation of Working Solutions

1) Prepare a working solution of **Wash Buffer** by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water (**ddH_2O**). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2) Prepare **20X ATP Solution** by adding **1.6 mL** of **ddH_2O** to the vial of **20X ATP** (lyophilized). Mix gently until dissolved. The final concentration of the **20X ATP Solution** should be **1.25 mM**. Store the solution in small aliquots (e.g. 200 µL) at -20℃.

3) Prepare **20X DTT Solution** by adding **0.25 mL** of **ddH_2O** to the vial of **20X DTT** (lyophilized). Mix gently until dissolved. The final concentration of the **20X DTT Solution** should be **200 mM**. Store the solution in small aliquots (e.g. 50 µL) at -20℃.

4) Prepare **10X GST-eIF2*α* Substrate Solution** by adding **0.6 mL** of **ddH_2O** to the vial of **GST-eIF2*α* Substrate** (lyophilized). Mix gently until dissolved. The final concentration of the **10X GST-eIF2*α* Substrate Solution** should be **62.5 µg/mL**. Store the solution in small aliquots (e.g. 100 µL) at -20°C.

5) Prepare **PKR Positive Control(s)**.

Catalytic Domain PKR

1st step: Dilute the **"PKR/EIF2 (Catalytic domain)"** (not provided. See the section "Materials Required but not Provided" above) with **"BSA-containing dilution buffer"** (not provided. See the section "Materials Required but not Provided" above) to be ~100 ng/µL.

2nd step: Dilute 1st step enzyme solution 1:5 with **Kinase Buffer**. Make sure a final concentration of the **PKR/EIF2 (Catalytic domain)** must be ~20 ng/µL.

Full Length PKR

Dilute **"PKR/EIF2 (Full length)"** (not provided. See the section "Materials Required but not Provided" above) with **Kinase Buffer** to be ~1 units/µL.

For Reference Purpose Only! Please refer to the user manual that came with your product.

6) Prepare **Reaction Buffers** for kinase reaction.

Reaction Buffer (Poly I:C minus)

For assays with **Catalytic Domain PKR**, prepare **Reaction Buffer (Poly I:C minus)** (Quantity required: 90 µL/assay). Mix following components. Discard any unused the buffer after use.

Components	96 assays	10 assays	1 assay
Kinase Buffer	8.0 mL	800 µL	80 µL
10X GST-eIF2α Substrate Solution	1.0 mL	100 µL	10 µL
20X DTT Solution	0.5 mL	50 µL	5 µL
20X ATP Solution	0.5 mL	50 µL	5 µL
Total	10 mL	1,000 µL	100 µL

Reaction Buffer (Poly I:C plus)

For assays with **Full Length PKR** or native PKR sample, prepare **Reaction Buffer (Poly I:C plus)** (Quantity required: 90 µL/assay). Mix following components. Discard any unused the buffer after use.

Components	96 assays	10 assays	1 assay
Kinase Buffer	8.0 mL	800 µL	80 µL
10X GST-eIF2α Substrate Solution	1.0 mL	100 µL	10 µL
20X DTT Solution	0.5 mL	50 µL	5 µL
20X ATP Solution	0.5 mL	50 µL	5 µL
5µg/mL poly I:C *	20 µL	2 µL	0.2 µL
Total	10.02 mL	1,002 µL	100.2 µL

* Not provided. See the section "Materials Required but not Provided" above

Reaction Buffer (ATP/ Poly I:C minus)

For assays of precise PKR activity, prepare **Reaction Buffer (ATP/Poly I:C minus)** (Quantity required: 90 µL/assay). Mix following components. Discard any unused the buffer after use.

Components	96 assays	10 assays	1 assay
Kinase Buffer	8.0 mL	800 µL	80 µL
10X GST-eIF2α Substrate Solution	1.0 mL	100 µL	10 µL
20X DTT Solution	0.5 mL	50 µL	5 µL
ddH_2O	0.5 mL	50 µL	5 µL
Total	10 mL	1,000 µL	100 µL

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2. Standard Assay

1) Remove the appropriate number of **Non-coated Microplate** and **Antibody-coated Microplate** wells from the pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2) Prepare all samples (diluted with **Kinase Buffer** as needed).

3) Add **10 µL** of **sample** and **PKR Positive Control** (See the "5) Prepare PKR Positive Control(s)." in "1. Preparation of Working Solution" above.) to each well of **Non-coated Microplate** on ice.

4) Start the kinase reaction by addition of **90 µL** of **Reaction Buffer*** to each well, cover with plate sealer or lid, and **incubate at 30°C for 60 minutes** shaking at ca. 300 rpm on an orbital microplate shaker.

* Select the suitable Reaction Buffer, Poly I:C plus or Poly I:C minus, for the type of sample and PKR Positive control. See the "6) Prepare Reaction Buffer for kinase reaction." in "1. Preparation of Working Solution" above.

5) Stop the kinase reaction by addition of **10 µL** of **EDTA Solution** to each well. Mix well by pipetting.

6) Transfer **100 µL** of the reaction mixture to each well of the **Antibody-coated Microplate**, cover with plate sealer or lid, and **incubate at room temperature (ca.25°C) for 60 minutes** shaking at ca. 300 rpm on an orbital microplate shaker.

7) Wash five times by filling each well with **Wash Buffer**. Remove residual Wash Buffer by gentle tapping or aspiration.

8) Pipette **100 µL** of **HRP-conjugated Detection Antibody** to each well, cover with plate sealer or lid, and **incubate at room temperature (ca.25°C) for 60 minutes** shaking at ca. 300 rpm on an orbital microplate shaker.

9) Wash five times by filling each well with **Wash Buffer**. Remove residual Wash Buffer by gentle tapping or aspiration.

10) Add **100 µL** of **Substrate Reagent** to each well and incubate **at room temperature (ca.25°C) for 5-20 minutes** shaking at ca. 300 rpm on an orbital microplate shaker.

11) Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

12) Measure absorbance in each well using a spectrophotometric plate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the plate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

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Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Note-2: Reliable signals are obtained when either O.D. values do not exceed 0.25 units for the blank (no enzyme control), or 2.5 units for Positive Controls.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of Positive Controls, perform a second reading at 405 nm. A new O.D. values, measured at 405 nm, is used to determine PKR activity of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

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3. Recommendations

I. Special considerations for screening inhibitors

In order to estimate the inhibitory effect on PKR activity in your test compounds correctly, it is necessary to conduct the control experiment of "Solvent Control Assay" at least once for every experiment and "Inhibitor Control Assay" at least once for the first experiment, in addition to "Test Compound Assay", as indicated in the following table. When test compounds cause an inhibitory effect on PKR activity, the level of "Test Compound Assay" weaken as compared with "Solvent Control Assay".

Inhibitor screening using Catalytic Domain PKR

Assay Reagents	Test Compound Assay	Solvent Control Assay	Inhibitor Control Assay
Reaction Buffer (Poly I:C minus) *	80 µL	80 µL	80 µL
Test Compound	10 µL	-	-
Solvent of Test Compound	-	10 µL	-
C16 (10 µM) **	-	--	10 µL
Catalytic Domain PKR ***	10 µL	10 µL	10 µL

* See the "6) Prepare Reaction Buffers for kinase reaction." in "1. Preparation of Working Solution" above.
** See the section "Materials Required but not Provided" above.
*** ~20 ng/µL of Catalytic Domain PKR. See the section "Materials Required but not Provided" and "5) Prepare Positive Control(s)" in "1. Preparation of Working Solution" above.

1) Following the table above, add the reagents to each well of the **Non-coated Microplate**. Finally, initiate reaction by adding 10 µL of **Catalytic Domain PKR** to each well and mixing thoroughly at room temperature. Cover with plate sealer or lid, and **incubate at 30°C for 60 minutes** shaking at ca. 300 rpm on an orbital microplate shaker.

2) Follow the step 5) to 12) of "2. Standard Assay" above.

Note: Although we suggest to conduct experiments as outlined in the table above, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user. Especially, an appropriate amount of the enzyme must be optimized by titration of the enzyme and setting the amount, which shows OD value does not exceed plateau range in dose response curve.

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Inhibitor screening using Full Length PKR or native PKR samples

Assay Reagents	Test Compound Assay	Solvent Control Assay	Inhibitor Control Assay
Reaction Buffer (Poly I:C plus) *	80 µL	80 µL	80 µL
Test Compound Solvent of Test Compound C16 (10 µM) **	10 µL - -	- 10 µL --	- - 10 µL
Full Length PKR *** (or native PKR sample)	10 µL	10 µL	10 µL

* See the "6) Prepare Reaction Buffers for kinase reaction" in "1. Preparation of Working Solution" above.
** See the section "Materials Required but not Provided" above.
*** ~1 units/µL of Full Length PKR. See the section "Materials Required but not Provided" and "5) Prepare Positive Control(s)" in "1. Preparation of Working Solution" above.

1) Following the table above, add the reagents to each well of the **Non-coated Microplate**. Finally, initiate reaction by adding 10 µL of **Full Length PKR** (or your native PKR sample) to each well and mixing thoroughly at room temperature. Cover with plate sealer or lid, and **incubate at 30°C for 60 minutes** shaking at ca. 300 rpm on an orbital microplate shaker.

2) Follow the step 5) to 12) of "2. Standard Assay" above.

Note: Although we suggest to conduct experiments as outlined in the table above, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user. Especially, an appropriate amount of the enzyme must be optimized by titration of the enzyme and setting the amount, which shows OD value does not exceed plateau range in dose response curve.

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II. Special considerations for measuring precise PKR activity

In order to measure the activity of PKR correctly, it is necessary to conduct the control experiment of "Inhibitor Control Assay" at least once for every experiment and "ATP Minus Control Assay" at least once for the first experiment, in addition to "No Enzyme Control Assay" as indicated in the following table. The level of A450 increases in "Test Sample Assay" when the sample contains PKR enzyme activity, although the high levels of A450 are not observed in "Inhibitor Control Assay", "ATP Minus Control Assay" and "No Enzyme Control Assay".

Assay Reagents	Test Sample Assay	Inhibitor Control Assay	ATP Minus Control Assay	Positive Control Assay	No Enzyme Control Assay
Reaction Buffer (Poly I:C plus or minus) *	80 µL	80 µL	-	80 µL	80 µL
Reaction Buffer (ATP/ Poly I:C minus) **	-	-	80 µL	-	-
C16 (10 µM) ***	-	10 µL	-	-	-
Solvent of C16 (10 µM)	10 µL	-	10 µL	10 µL	10 µL
native PKR sample	10 µL	10 µL	10 µL	-	-
PKR Positive Control ****	-	-	-	10 µL	-
Buffer of native PKR sample	-	-	-	-	10 µL

* Select the suitable Reaction Buffer, Poly I:C plus or Poly I:C minus, for the type of PKR Positive control. See the "6) Prepare Reaction Buffers for kinase reaction." in "1. Preparation of Working Solutions" above.
** See the "6) Prepare Reaction Buffers for kinase reaction." in "1. Preparation of Working Solutions" above.
*** See the section "Materials Required but not Provided" above.
**** ~20 ng/µL of Catalytic Domain PKR or ~1 units/µL of Full Length PKR. See the section "Materials Required but not Provided" and "5) Prepare Positive Control(s)" in "1. Preparation of Working Solution" above.

1) Following the table above, add the reagents to each well of the **Non-coated Microplate**. Finally, initiate reaction by adding 10 µL of your **native PKR sample** or **PKR Positive Control** or **Buffer of native PKR sample** to each well and mixing thoroughly at room temperature. Cover with plate sealer or lid, and **incubate at 30°C for 60 minutes** shaking at ca. 300 rpm on an orbital microplate shaker.

2) Follow the step 5) to 12) of "2. Standard Assay" above.

Note: Although we suggest to conduct experiments as outlined in the table above, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user. Especially, an appropriate amount of the enzyme must be optimized by titration of the enzyme and setting the amount, which shows OD value does not exceed plateau range in dose response curve.

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Evaluation of Results

Average the absorbance values for the Positive Control and all experimental sample duplicate values (when applicable). When **Catalytic Domain PKR** (See the "5) Prepare Positive Control(s)" of "1. Preparation of Working Solution" in the section "Detailed Protocol" above) is included in 200 ng/assay as an internal control for the kinase reaction, the absorbance value should be greater than 1.0 with a background less than 0.25 when using Reaction buffer.

Assay Characteristics

The CycLex Research Product **CycLex PKR/EIF2AK2 Kinase Assay Kit** has been shown to detect the activity of purified PKR and column fractions containing PKR. The assay may be used to follow the purification of PKR or can be used to detect PKR kinase activity in the immunoprecipitate from cell lysates using anti-PKR antibody.

Troubleshooting

1. All samples and controls should be assayed in duplicate, when a standard assay is being performed, using the protocol described in the section "Detailed Protocol". Incubation times or temperatures significantly different from those specified may give erroneous results.

2. The reaction curve is nearly a straight line if the kinetics of the assay is of the first order. Variations in the protocol can lead to non-linearity of the curve, as can assay kinetics of other than first order. For a non-linear curve, point to point or quadratic curve fit methods should be used.

3. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the "Detailed Protocol" were followed accurately, such results indicate a need for washer maintenance.

4. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the CycLex Research Product **CycLex PKR/EIF2AK2 Kinase Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt kit reagents should be stored at 4°C. Antibody-coated Microplate should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

For research use only, not for use in diagnostic or therapeutic procedures

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Sample Preparation

Numerous extraction and purification methods can be used to isolate PKR. The following protocols have been shown to work with a number of different cells and enzyme sources and are provided as examples of suitable methods. Crude samples can frequently be used without dilution while more concentrated or highly purified PKR should be diluted. It is strongly advised that the user always perform an initial experiment to determine the proper dilution to be used in subsequent experiments. This need not be any more than a single time point assay using serial dilutions of the crude extract, cell lysate or sample fraction taken prior to a purification step. One eight well strip of the plate should be sufficient for this initial experiment. All sample preparation should be performed at 4°C and recovered fractions should be kept at 4°C to prevent loss of enzymatic activity.

CAUTION: It should be noted that this assay kit detects not only PKR activity but also other protein kinases in crude extracts and column samples. The presence of PKR protein in the samples should be traced by other methods, e.g. western blotting.

Immunoprecipitation Protocol Followed by Measuring PKR Activity

1. Preparation of Solution and Reagent

1) Cell Lysis Buffer
20 mM Tris HCl, pH 7.5, 250 mM NaCl, 10% glycerol, 0.5% Nonidet® P-40, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 µg/mL pepstatin, 0.5 µg/mL leupeptin, 5 mM NaF, 2 mM Na_3VO_4, 2 mM β-glycerophosphate, 1mM DTT, 1x Phosphatase Inhibitor Cocktail

2) Protein A Agarose Beads (50% bead slurry)
Add 5 mL of 1X PBS to 1.5 g of protein A agarose beads. Shake 2 hours at 4°C; spin down. Wash beads twice with PBS. Resuspend beads in 1 volume of PBS. (Can be stored for 2 weeks at 4°C)

3) Poly-L-lysine-coated Plate
Coat the plate with 25 µg/mL poly-L-lysine (PLL) in PBS for 4-12 h at 37°C. Subsequent to a washing step with PBS.

2. Treatment of Cells

1) Plate adherent cells in PLL-coated 6-well plate dish at ~1 x 10^6 cells/plate.

2) Incubate the culture dish at 37°C for 12-16 hours in CO_2 incubator.

3) Change the medium to fresh media containing 10 µg/mL poly I:C (See the section "Materials Required but not Provided").

4) Incubate the culture dish at 37°C for appropriate time.

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3. Preparing Cell Lysates

1) Remove media, and wash cells with ice-cold PBS and aspirate.

2) Add 1 mL of ice-cold Cell Lysis Buffer to each plate (6 well-plate dish) and shake at ca. 300 rpm on an orbital microplate shaker for 120 minutes each at 4°C.

3) Scrape off and transfer the lysate to microcentrifuge tubes and microcentrifuge at 15, 000 rpm for 10 minutes at 4°C,

4) Transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, the lysate can be stored at -70°C.

4. Immunoprecipitation and assay kinase activity

1) Take 100 µL of the cell lysate and add anti-human PKR polyclonal antibody (CycLex Co., Ltd., Cat#CY-P1043; 1-2 µg) and incubate with gentle rocking for 1–3 hours at 4°C.

2) Add 20 µL of Protein A Agarose Beads (50% bead slurry) and incubate with gentle rocking for 1–3 hours at 4°C.

3) Microcentrifuge for 30 seconds at 4°C. Wash the protein A agarose beads 3 times with 500 µL of Cell Lysis Buffer and once with Kinase Buffer. Keep on ice during the washes.

4) Resuspend the protein A agarose beads with 20-40 µL of Kinase Buffer and use 10 µL as an enzyme sample* to measure PKR activity according to the procedure in the step 3) to 12) of "2. Standard Assay" in the section "Detailed Protocol" above.

* *Please take care to transfer the protein A agarose beads to the wells of the* ***Antibody-coated Microplate*** *as little as possible.*

Note: Although we suggest to conduct experiments as outlined above, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user. Especially, an appropriate cell lysate must be optimized by titration of the cell lysate and setting the amount, which shows OD value does not exceed plateau range in a dose-response curve. **NO WARRANTY OR GUARANTEE OF PERFORMANCE USING THESE PROCEDURES IS MADE OR IMPLIED.**

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Example of Test Results

Fig.1 Dose dependency of Catalytic Domain PKR

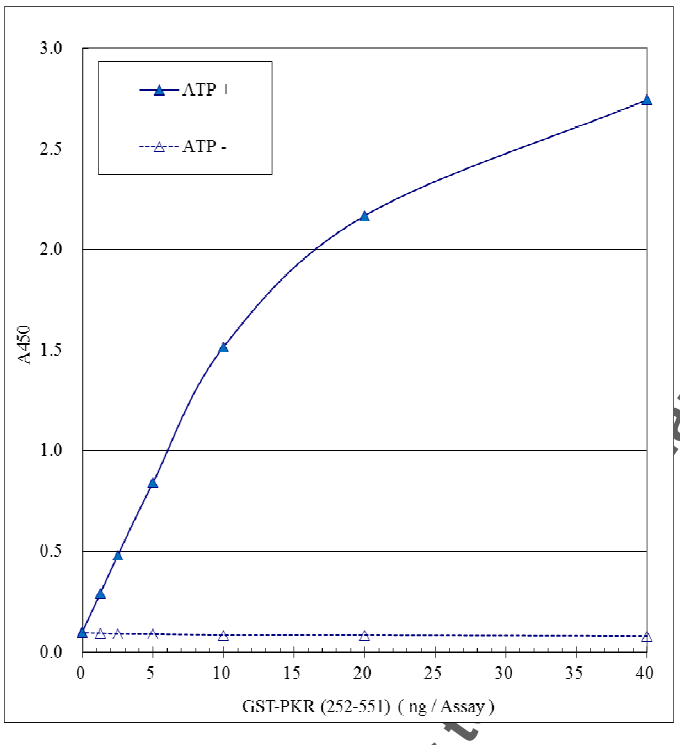

Fig.2 Km for ATP of Catalytic Domain PKR

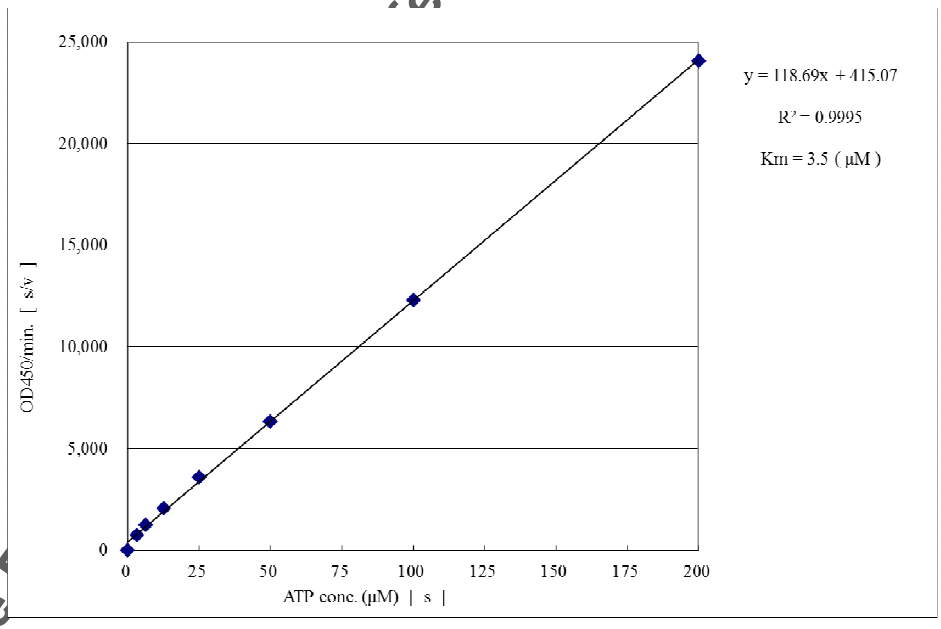

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Fig.3 Effect of NaCl on the kinase activity of Catalytic Domain PKR

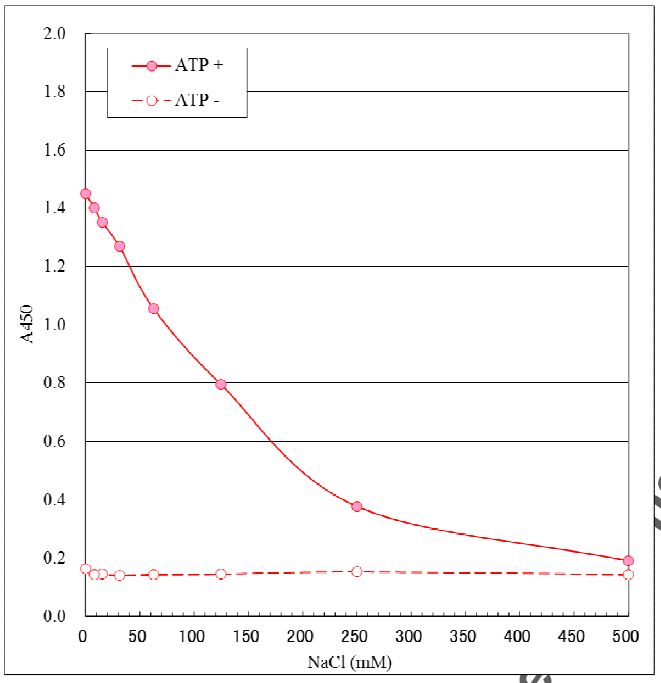

Fig.4 Effect of C16, a PKR inhibitor, on the kinase activity of Catalytic Domain PKR

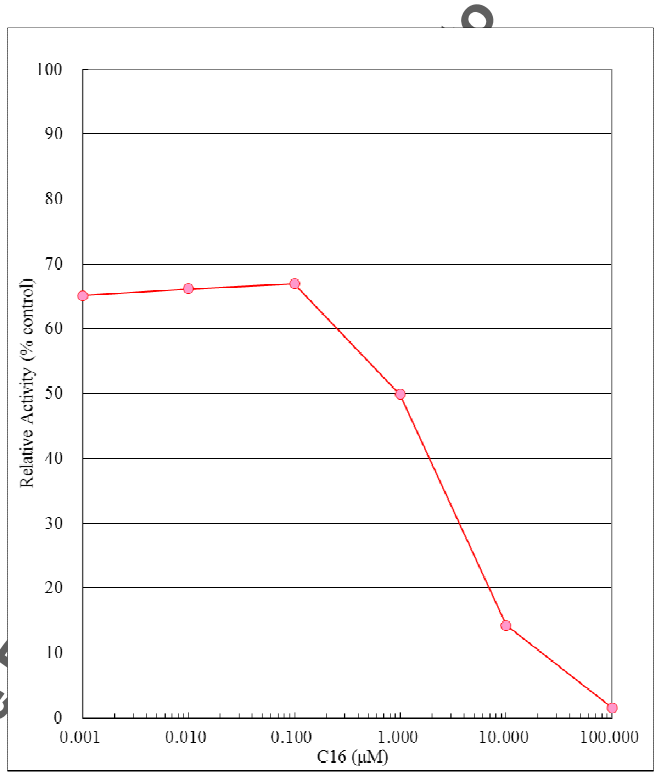

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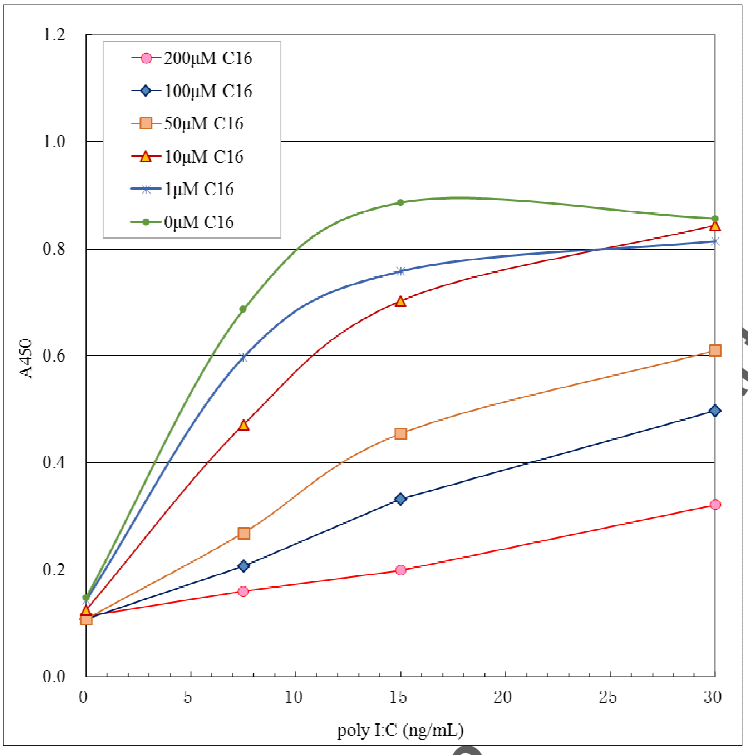
Fig.5 Effect of poly I:C and C16 on the kinase activity of Full Length PKR



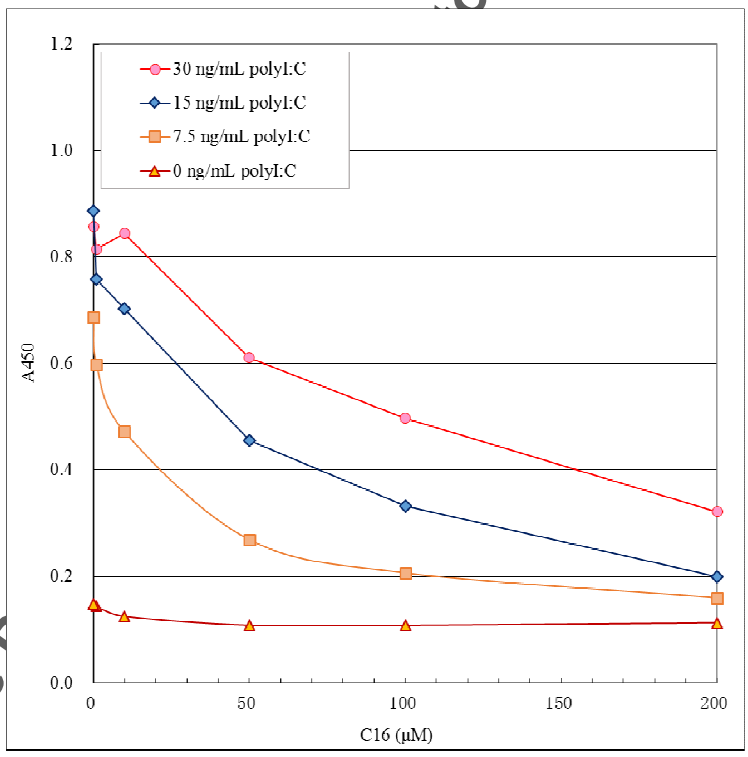


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Fig.6 Effect of poly I:C on the kinase activity of Full Length PKR

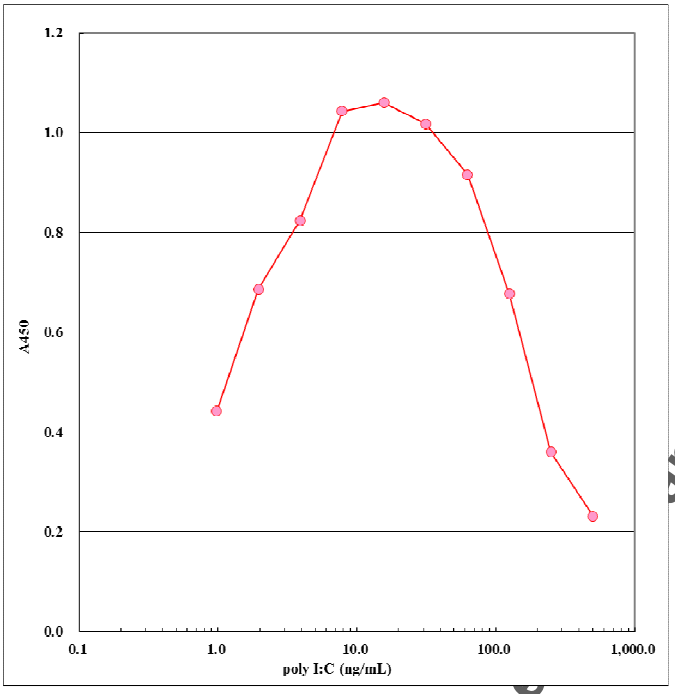
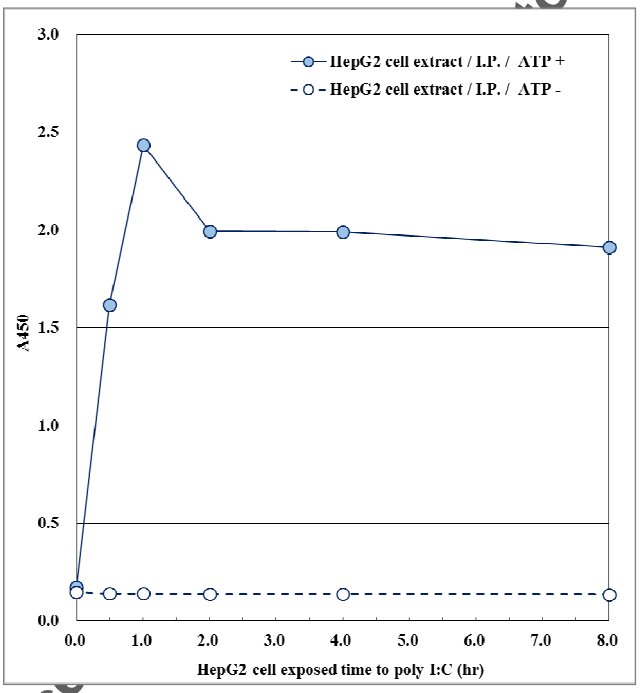


Fig.7 Time course of poly I:C effect on native PKR immunoprecipitated from HepG2 cell



Procedures as a guideline

Plate HepG2 cells at a density of 0.5×10^6 cells /well in 6-well plate.

↓ Culture at 37°C, O/N

Add poly I:C to final conc. of 10 µg/mL to each well.

↓ Culture at 37°C for indicated times

Remove the medium.

↓

Gently wash the cells with ice cold PBS.

↓

Discard PBS.

↓

Add 1 mL of Cell lLysis Buffer to each well.

↓ Incubate at 4°C for 2 hour with gentle shaking

Harvest the lysates.

↓ Centrifuge at 13,000 rpm for 5 minutes at 4°C

Use 100 µL of the clear lysate as a sample for IP-kinase assay.

For Reference to the manual that came with your product.

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